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Mesenchymal Expression of Nuclear Factor-κB Inhibits Epithelial Growth and Branching in the Embryonic Chick Lung

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It is becoming increasingly recognized that the ubiquitous, inducible transcription factor nuclear factor-κB (NF-κB) is involved in developmental processes. For example, NF-κB acts as a mediator of epithelial–mesenchymal interactions in the developing chick limb. We investigated the role of NF-κB in directing the branching morphogenesis of the developing chick lung, a process which relies on epithelial–mesenchymal communication. High level expression of *relA* was found in the mesenchyme surrounding the nonbranching structures of the lung but was not detected either in the mesenchyme surrounding the branching structures of the distal lung or in the developing lung epithelium. Specific inhibition of mesenchymal NF-κB in lung cultures resulted in increased epithelial budding. Conversely, expression of a *trans*-dominant activator of NF-κB in the lung mesenchyme repressed budding. Ectopic expression of RelA was sufficient to inhibit the ability of the distal mesenchyme to induce epithelial bud formation. Cellular proliferation in the mesenchyme was inhibited by hyperactivation of NF-κB in the mesenchyme of lung cultures. Interestingly, increased NF-κB activity in the mesenchyme also decreased the proliferation of the associated epithelium, while inhibition of NF-κB activity increased cellular proliferation in lung cultures. Expression patterns of several genes which are known to influence lung branching morphogenesis were altered in response to changes in mesenchymal NF-κB activity, including *fgf10, bmp-4,* and *tgf-β1.* Thus NF-κB represents the first transcription factor reported to function within the lung mesenchyme to limit growth and branching of the adjacent epithelium.

INTRODUCTION

The developing lung is an ideal system for the study of molecular mechanisms that regulate branching morphogenesis. The lung develops in a well-defined spatial and temporal pattern, with the epithelium originating from the ventral portion of the foregut as an epithelial evagination that invades the surrounding splanchnic mesenchyme. Epithelial growth and morphogenesis require the presence of the mesenchymal component of the lung, and the characteristic morphology of the lung is determined by the nature of the surrounding mesenchyme (Rudnick, 1933). For example, pulmonary mesenchyme from the distal tips of the developing lung induces branching of the distal epithelium. Moreover, when ectopically placed in the context of tracheal epithelium, this mesenchyme can induce budding of the tracheal epithelium and differentiation of the epithelium toward a more distal phenotype (Alescio and Cassini, 1962; Shannon *et al.*, 1994). Conversely, tracheal mesenchyme inhibits epithelial branching when placed in the context of the pulmonary epithelium (Wessells, 1970). This suggests that the distal mesenchyme supplies molecular cues to the epithelium, which results in the branching of the distal epithelium. Recent evidence suggests that a number of secreted factors produced in the embryonic lung regulate epithelial growth or branching, including fibroblast growth factors (FGF) 7 and 10 (Post *et al.*, 1996; Bellusci *et*

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al., 1997; Cardoso *et al.,* 1997; Ohuchi *et al.,* 1997), transforming growth factor-β (TGF-β; Serra *et al.,* 1994; Zhao *et al.,* 1996; Hogan, 1997), bone morphogenetic protein 4 (BMP-4; Bellusci *et al.,* 1996), sonic hedgehog (SHH; Urase *et al.,* 1996; Bellusci *et al.,* 1997; Pepicelli *et al.,* 1998; Litingtung *et al.,* 1998), hepatocyte growth factor/scatter factor (Ohmichi *et al.,* 1998), and epidermal growth factor (Goldin and Opperman, 1980; Threadgill *et al.,* 1995; Miettinen *et al.,* 1997). Many of these factors may induce branching of the developing lung epithelium, as is observed in the distal portion of the lung, suggesting that the lack of these inductive factors from the proximal mesenchyme may account for the lack of branching in the proximal or tracheal epithelium. Additionally, active inhibition of epithelial branching by the tracheal mesenchyme may be partially responsible for the lack of branching in the tracheal epithelium. TGF-β1, which is normally synthesized by the mesenchyme surrounding the proximal structures of the embryonic lung and at cleft points of branches, inhibits epithelial branching in mouse lung cultures (Serra *et al.,* 1994). Transgenic mice that ectopically express TGF-β1 in the embryonic distal lung epithelium via the *surfactant protein-c* (*SP-C*) gene enhancer/promoter display a decrease in branching and arrested development at the pseudoglandular stage (Zhao *et al.,* 1996). It is thought that TGF-β1 represses epithelial bud formation by decreasing the rate of cellular proliferation of the embryonic lung epithelium and inhibiting the expression of N-myc, a transcription factor that is required for the proliferation of the embryonic mouse lung epithelium (Serra *et al.,* 1995).

The transcription factor family known as nuclear factor-κB (NF-κB) has been studied predominantly in regard to B- and T-lymphocyte physiology. Roles for NF-κB in directing gene expression, cell proliferation, and apoptosis have been established for these cell types (Verma *et al.,* 1995; Attar *et al.,* 1998; Ghosh *et al.,* 1998). Five known vertebrate NF-κB family members have been identified (c-Rel, RelA, RelB, p50, and p52), all of which share a conserved structural motif composed of approximately 300 amino acids, known as the Rel-homology domain. This domain is responsible for DNA binding, dimerization, nuclear localization, and protein interactions (reviewed in Verma *et al.,* 1995; Thanos and Maniatis, 1995). These NF-κB family members exist in the cytoplasm as homo- or heterodimers, bound to a class of inhibitory proteins known as the IκBs (for inhibitors of -κB). Seven vertebrate IκB family members have been identified to date, including IκB-α, IκB-β, IκB-ϵ, IκB-γ, bcl-3, p100, and p105 (Whiteside *et al.,* 1997). Upon stimulation by specific signals, such as cytokines or growth factors, several IκBs may be phosphorylated on invariant serine residues located in the amino-terminus of the protein, by a kinase complex known as IκB-kinase (IKK; Mercurio *et al.,* 1997). This phosphorylation induces its degradation through the ubiquitin–proteasome pathway, allowing NF-κB to translocate to the nucleus, bind DNA in a sequence-specific manner, and influence target gene expression (Beg *et al.,* 1993). Although the role of NF-κB in lymphocyte development is well characterized, relatively little is known about the requirements of NF-κB during organogenesis. However, recent evidence demonstrates that NF-κB is critical in the developing limb for mediating an interaction between the epithelium and the mesenchyme (Bushdid *et al.,* 1998; Kanagae *et al.,* 1998). Specifically, expression of c-Rel in the limb mesenchyme requires the signals from the apical ectodermal ridge (AER), a specialized epithelial structure overlying the limb bud mesenchyme that directs outgrowth of the developing limb. Beads soaked in FGF-2 or FGF-4, or viruses that express FGF-2 or -4, can functionally replace the AER (Laufer *et al.,* 1994: Ohuchi *et al.,* 1997). Moreover, FGF-2 or -4 are sufficient to maintain expression of c-Rel in the absence of the AER (Bushdid *et al.,* 1998; Kanagae *et al.,* 1998). In the absence of NF-κB activity, expression of the limb morphogens *shh* and *bmp-4* was perturbed (Bushdid *et al.,* 1998; Kanagae *et al.,* 1998). Furthermore, mesenchymal expression of NF-κB is required in the limb to maintain the integrity of the overlying epithelium, since the inhibition of NF-κB in the limb mesenchyme caused aberrant morphology of the AER.

The development of the lung shows marked similarities to that of the limb. Both processes utilize many of the same signals to direct morphogenesis of the developing lung. Specifically, FGFs, BMP-4, and SHH regulate branching and proliferation of the lung epithelium (Bellusci *et al.,* 1997a,b; reviewed in Hogan, 1999). The molecular parallels that exist between the epithelial–mesenchymal interactions occurring in the limb and the lung are quite striking and suggest that NF-κB may be required in the lung to mediate epithelial–mesenchymal interactions, directing gene expression that ultimately regulates cell growth or differentiation. We tested this hypothesis by altering NF-κB activity in cultures of embryonic chick lungs to determine the effect on gene expression patterns, growth, and branching in the developing lung. Our results suggest that NF-κB activity in the embryonic chick lung mesenchyme inhibits branching of underlying epithelium.

MATERIALS AND METHODS

Embryo incubation. Fertilized chicken eggs (SPAFAS; Charles Rivers Laboratories) were incubated at 39°C in a humidified incubator and rotated daily.

Whole-mount in situ hybridization. Embryonic chick lungs were harvested from stage 23–32 embryos in ice-cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS. Alternatively, virally infected lung cultures were immediately fixed in 4% PFA 72 h after infection. The cDNA sequences of the genes of interest were subcloned into pBluescript SK(−) (Stratagene), the plasmids were linearized, and the sense and antisense riboprobes were synthesized from the T3 or T7 transcription initiation sites using 5 U of T3 or T7 RNA polymerase (Promega, Madison, WI). The sequences subcloned into pBluescript were a 1.1-kb *Sac*I fragment from the avian *relA* cDNA and a 460-bp *Bam*HI fragment from the avian *tgf-β1* cDNA. Subcloning of the avian *bmp-4* cDNA, the murine *fgf10* cDNA, the murine *twist*

cDNA, and the avian *shh* cDNA is described elsewhere (Bushdid *et al.*, 1998; Bellusci *et al.*, 1997a). Fixed lungs were probed with the indicated antisense and sense digoxigenin-labeled riboprobes as previously described (Bushdid *et al.*, 1998). Hybridization of the probe was visualized histochemically using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim). Lungs were postfixed in 4% PFA and photographed or snap frozen in OCT freezing compound (Finetek U.S.A., Inc., Torrance, CA) and cryosectioned at 50 μm.

Electrophoretic mobility shift assay. Nuclear extracts were prepared from stage 27 lungs or from the proximal portion or the distal portion of stage 32 embryonic chick lungs. The harvested lungs were frozen and pulverized in liquid nitrogen, lysed in buffer A [3 ml; 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 25 μg/ml PMSF] on ice for 10 min, pelleted, and resuspended in buffer A (1 ml) supplemented with 0.05% Nonidet P-40 and homogenized using a 25-gauge syringe needle. The nuclei were pelleted, resuspended in buffer B (0.25 ml; 5 mM Hepes, pH 7.9, 26% glycerol v/v, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 300 mM NaCl, and 25 μg/ml PMSF), incubated on ice for 40 min, and pelleted. The supernatants containing nuclear extracts were stored at −80°C. Complementary annealed oligonucleotides encompassing the NF-κB binding element of the human immunodeficiency virus long terminal repeat (HIV-LTR) (plus strand 5′-GAT CGA GGG GAC TTT CCC TAG C-3′) or consensus SP-1 binding sequences (5′-GAT CGA TCG GGG CGG GGC GAT C-3′) were end-labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase. Both oligonucleotides were from Stratagene Gel Shift Assay Kit (Stratagene, Inc., La Jolla, CA). Nuclear extracts (10 μg) were incubated in the presence of binding buffer [20 mM Hepes, pH 7.9, 15% glycerol, 5 mM KCl, 0.1 mM EDTA, 0.2 μg/ml poly(dI–dC)] on ice for 10 min. Probe (30,000 cpm) was added and allowed to incubate for 30 min. Reactions were run on a 4% polyacrylamide gel at room temperature in 0.25× TBE at 150 V. Gels were dried and exposed to autoradiographic film overnight at −80°C.

Virus preparation and infection. The proviral RCAS-alkaline phosphatase and RCAS-αΔN vectors were prepared as described (Bushdid *et al.*, 1998). To create RCAS-relA, a 1.9-kb *Bss*HI/*Xho*I fragment encoding the full-length chick RelA cDNA was subcloned into a *Cla*12–*Nco* adaptor plasmid and then into RCAS-BP(A) (Morgan and Fekete, 1996; Hughes, 1987). Creation of RCAS-IKK50 was achieved by cloning the full-length, FLAG-tagged IKK-2 (S177A, S181A) cDNA (a generous gift from Dr. Frank Mercurio, Signal Pharmaceuticals; Mercurio *et al.*, 1997) into *Cla*12–*Nco* and then into RCAS-BP(A). RCAS viral stocks were produced and concentrated as described (Bushdid *et al.*, 1998). Viral infection of the lung mesenchyme was achieved by bathing the lungs at 37°C, 5% CO_2 for 1 h in a concentrated stock (100 μl) of the RCAS virus. Expression of alkaline phosphatase was confirmed in whole mount by alkaline phosphatase enzymography, using a BCIP/NBT substrate (Promega), according to the manufacturer's instructions to produce a colorimetric precipitate. Expression of IκB-αΔN, IKK50, and RelA was confirmed by Western analysis (see below).

Transfection of CEFs and analysis of reporter gene expression. Primary chick embryonic fibroblasts were cultured in DMEM supplemented with 10 μg/ml gentamicin sulfate, 4 mM L-glutamine, 2% heat-inactivated chick serum, and 10% heat-inactivated fetal calf serum. The cells were infected with RCAS-alkaline phosphatase, RCAS-αΔN, RCAS-IKK50, or RCAS-relA by incubation in the presence of infectious particles for 48 h. The infected cells were transfected by the calcium phosphate method (Muraoka *et al.*, 1999), with an NF-κB-responsive reporter construct composed of sequences derived from the HIV-LTR (Kretzshmar *et al.*, 1992; Doerre *et al.*, 1993), driving expression of the *Photinus* luciferase cDNA (pGL2 basic;). These cells were treated with or without recombinant human TNF-α (20 ng/ml; R & D Systems) for 6 h, then crude whole-cell extracts were harvested in cell lysis buffer (Analytical Luminescence Laboratory, Ann Arbor, MI) according to the manufacturer's instructions. Cell extracts (10 μg total cellular protein) were directly used for enzymatic luciferase assay. Cotransfection with an expression vector containing the *lacZ* gene driven by the CMV promoter was used to normalize for transfection efficiency, as previously described (Muraoka *et al.*, 1999).

Western analysis. Chick embryonic fibroblasts were collected and resuspended in 0.25 M Tris, pH 7.5, 18 h after viral infection. Whole-cell extracts were obtained using three freeze–thaw cycles, followed by centrifugation at 12,000*g* for 20 min at 4°C. The supernatant containing the cell extract was assayed for protein content by Lowry Analysis (Bio-Rad Protein Assay Kit) and then snap frozen and stored at −80°C. Cell extracts (30 μg) were denatured in SDS–Laemmli loading buffer (20 μl), heated to 90°C for 2 min, then resolved on a 10% reducing SDS–polyacrylamide gel. The resolved proteins were transferred to PVFD membranes (Immobilon) in 25 mM Tris–HCl, 192 mM glycine, and 20% methanol at 4°C for 3 h at 250 mA. The blots were incubated with one of the following antibodies: a mouse monoclonal anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO), a rabbit anti-human p65 polyclonal antibody (Santa Cruz Biotechnologies Inc., Santa Cruz, CA), or a rabbit anti-human IκB-α polyclonal antibody, directed against the carboxyl-terminus of the IκB-α protein (Santa Cruz Biotechnologies, Inc.). The membranes were incubated with the antibody and then incubated with a horse universal anti-IgG antibody, followed by incubation with an avidin–biotin horseradish peroxidase complex (Vectastain, Vector Laboratories, Burlingame, CA). A chemiluminescent visualization system (NEN Life Sciences Products) was applied according to the manufacturer's protocol, and the membranes were exposed to autoradiographic film.

Lung cultures and epithelial–mesenchymal recombinations. Embryonic chick lungs were harvested at stage 27 in ice-cold PBS, pH 7.4. Lungs were cultured on Nucleopore polycarbonate filters (8 μm pore size; Corning Separations Division) at the air–liquid interface in a defined medium containing Dulbecco's modified Eagle's medium:F12 (DMEM:F12) supplemented with 1 μg/ml BSA, 10 μg/ml gentamicin sulfate, 4 mM L-glutamine, and 50 ng/ml human FGF-7 (all from Sigma). Lungs were cultured in 5% CO_2 in a humidified incubator at 37°C for 72–120 h. Photographic documentation of growth and branching of the cultured lungs was taken at 24, 48, and 72 h following viral infection, at which point the total number of lung buds per lung was determined. The lung epithelium was separated from the mesenchyme by incubating the distal tips or the trachea in dispase (Collaborative Biomedical Products, Bedford, MA) for 30 min at 37°C and washing in DMEM: F12 with 10% fetal calf serum. The mesenchyme and epithelium were separated by microscopic dissection using ultrafine tungsten needles. The epithelial rudiments were incubated with dispase for an additional 10 min to remove any residual mesenchyme and then stored on ice until recombination. The mesenchyme was infected as described above. The epithelial and mesenchymal components were recombined on Nucleopore filters.

Reverse transcription-polymerase chain reaction. Embryonic chick lungs were harvested at the indicated stages. Lungs were immediately utilized for RNA isolation or were cultured for 24 or 72 h, as described above, at which point the cultured lungs were utilized for total RNA isolation. Freshly harvested stage 27 lungs were separated into distal and proximal epithelial and mesenchymal com-

ponents. These specific tissues were utilized for total RNA isolation. Total cellular RNA was isolated using TRIzol reagent (Gibco BRL). Total cellular RNA was reverse transcribed using oligo(dT) primer and AMV reverse transcriptase (Ambion), as previously described (Ohuchi *et al.*, 1997; Riddle *et al.*, 1993; Pasquale, 1992; Domdey *et al.*, 1983). PCR analysis of gene expression was performed using 25-mer oligonucleotides corresponding to the following cDNA sequences: chick *tgf-β1* [primer 1 (nt 817–839, 5′ atgagaagaactgctgcgtgcggcc 3′) and primer 2 (nt 962–986, 5′ ttggttgtacagcgccagcaccttg 3′)], chick *fgf10* [primer 1 (nt 504–528, 5′ ttacaacaaatactttctcaagatc 3′) and primer 2 (nt 661–686, 5′ tccttagagccgtagacttttcctt 3′); Ohuchi *et al.*, 1997], chick *sonic hedgehog* [primer 1 (nt 788–812, 5′ gtcaaagcagaaaactcagtggcag 3′) and primer 2 (nt 935–959, 5′ ggaaggtgaggaagtcactgtagag 3′); Riddle *et al.*, 1993], chick *cek3/fgfr2* [primer 1 (nt 1801–1825, 5′ aatatcatcaatcttcttggagcct 3′) and primer 2 (nt 2072–2096, 5′ tctgctattttcatgacgttatttt 3′); Pasquale, 1992], and chick *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) [primer 1 (nt 800–824, 5′ attcctccacctttgatgcgggtgc 3′) and primer 2 (nt 964–988, 5′ ctccaacaaagggtcctgcttccct 3′); Domdey *et al.*, 1983]. All PCRs were performed over 30 cycles of the following temperatures and incubation times: 94°C for 1 min, 59°C for 1 min, 72°C for 30 s. PCRs were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and photodocumented.

Bromodeoxyuridine (BrdU) labeling and detection. Lungs were cultured for 48 h as described above, then BrdU labeling reagent (Cell Proliferation Kit; Amersham) was diluted 1:1000 in the defined culture medium and incubated for 6 h. Lungs were immediately fixed in 4% PFA. BrdU incorporation was detected by whole-mount immunohistochemistry using the Zymed Streptavidin–Biotin BrdU Staining Kit (Zymed Laboratories, Inc., San Francisco, CA) according to the manufacturer's instructions. Unlabeled nuclei were counterstained with hematoxylin, and frozen sections (5–7 μM) were used for analysis.

RESULTS

The role of NF-κB in lung development was examined using the developing chick lung as a model system. The expression of *relA*, a major transcriptional activator of the NF-κB family, was examined by whole-mount *in situ* hybridization. A diffuse pattern of *relA* expression was observed throughout the chick lung mesenchyme at stages 23 through 27 of embryogenesis (Hamburger and Hamilton, 1951), but *relA* was not detected in the epithelium at these stages (Fig. 1a). Beginning at stage 28, intense *relA* expression became highly restricted to the proximal mesenchyme surrounding the trachea and primary bronchi, but was absent from the distal lung mesenchyme (Fig. 1b). By stage 30, expression of *relA* was detected in the mesenchyme surrounding the developing cervical air sac, a specialized, nonbranching structure of the avian lung, and was maintained in the mesenchyme of other nonbranching structures (Figs. 1c–1e). Mesenchymal expression of *relA* in proximal areas of the embryonic chick lung was detected through stage 32 (Fig. 1f). In contrast, *cek3*, the chick *fgf receptor 2* (*fgfr2*) homologue, was expressed specifically in the epithelium at stage 27 (Fig. 1g). Epithelial expression was expected for *cek3*, since *fgfr2* is a marker for lung epithelium in embryonic mammalian lungs (Peters *et al.*, 1992, 1994). Through stage 32, *cek3* mRNA expression was predominantly found in the epithelium of the embryonic chick lung (Fig. 1h). Cross section of a stage 28 trachea confirmed the specific expression of *relA* in the mesenchyme and its absence from the embryonic lung epithelium (Fig. 1i). Sections of stage 28 lungs confirmed that *cek3* mRNA is restricted to the embryonic chick lung epithelium (Fig. 1j). This dynamic spatial and temporal expression of *relA* suggests that NF-κB might be involved in the development of the chick lung.

Biochemical approaches have shown that NF-κB typically resides in the cytoplasm in an inactive state and is transported to the nucleus only upon activation (Verma *et al.*, 1995). To resolve the status of NF-κB activity in the developing chick lung, nuclear extracts were harvested from stage 27 and stage 32 chick lungs and utilized in electrophoretic mobility shift assays. Nuclear extracts from stage 27 lungs produced a mobility shift of κB1, a labeled oligonucleotide encompassing the NF-κB binding region of the HIV-LTR enhancer (Fig. 2a, lane 2). Competition using a 100-fold excess of unlabeled κB1 eliminated binding of the labeled probe to nuclear proteins (data not shown). Stage 32 lungs were separated into proximal (nonbranching) and distal (branching) components prior to the isolation of nuclear extracts. A specific mobility shift was produced by proximal nuclear extracts (Fig. 2a, lane 3), suggesting that NF-κB is present in the nuclei of cells within the proximal regions of the embryonic chick lung. Although a specific complex was also observed in nuclear extracts taken from the distal portion of stage 32 lungs, the intensity of the shifted species was greatly reduced compared to that produced by proximal nuclear extracts, even though identical amounts of nuclear proteins were used (Fig. 2a, lane 4). An oligonucleotide encompassing binding sites for the ubiquitous transcription factor SP1 was shifted with equal intensities by nuclear extracts from stage 27, proximal stage 32, or distal stage 32 embryonic chick lungs (Fig. 2a, lanes 6–8). These results are consistent with the expression patterns of *relA* mRNA and suggest that NF-κB exhibits nuclear binding activities within the proximal chick lung mesenchyme, which would be predicted to influence gene expression.

In order to determine the biological significance of NF-κB during lung morphogenesis, activity of NF-κB was specifically inhibited by expression of a *trans*-dominant inhibitor of NF-κB, known as IκB-αΔN (Bushdid *et al.*, 1998). This mutant isoform of avian IκB-α lacks 40 amino-terminal amino acids, which include the signal-responsive phosphorylation and ubiquitination domains. This mutation prevents the ubiquitin-mediated degradation of IκB-α, which blocks translocation of NF-κB to the nucleus and effectively inhibits the activity of NF-κB (Bushdid *et al.*, 1998). Alternatively, NF-κB was hyperactivated by expression of IKK50, a constitutively active isoform of human IKK-2, in which two serine phosphorylation sites are mutated to glutamic acid (S172E, S177E), mimicking a state of constant phosphorylation (Mercurio *et al.*, 1997). IκB-αΔN, IKK50, wild-type avian RelA, and placental alkaline phosphatase were packaged into the proviral vector of a replication-competent

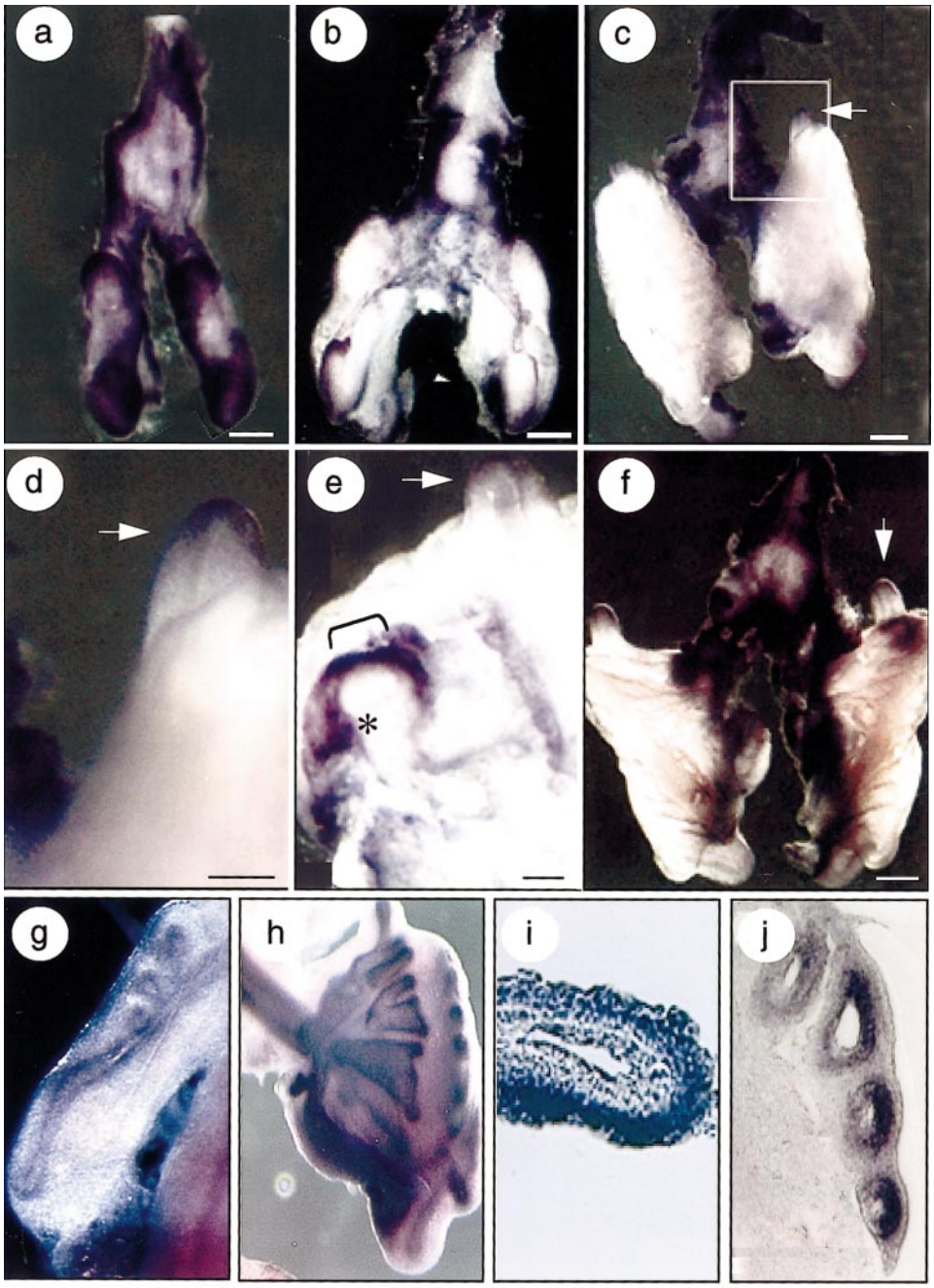

FIG. 1. Expression of *relA* mRNA in discrete spatial and temporal patterns in the developing chick lung. Whole-mount *in situ* hybridization for avian *relA* mRNA was performed on lungs isolated from chick embryos at stages 27 (a), 28 (b), 30 (c, d, e), and 32 (f). (a) Transcripts of *relA* were detected in a diffuse pattern throughout the mesenchyme. (b) *relA* mRNA expression detected in the proximal mesenchyme and in the distal aspects of the pulmonary mesenchyme. (c) *relA* mRNA expression intensified surrounding the proximal nonbranching structures and in the cervical air sacs. Arrow points to a cervical air sac. Boxed area indicates area of magnification shown in (d). (d) Increased magnification of (c). Arrow indicates expression of *relA* in the mesenchyme of the cervical air sac. (e) Primary bronchus (brackets) from stage 30 lung was transversely bisected to demonstrate detection of *relA* transcripts in the mesenchyme but not in the epithelium. Asterisk indicates the epithelium of the primary bronchus; brackets indicate the mesenchyme of the primary bronchus. (f) Intense *relA* expression at stage 32. Arrow indicates cervical air sac. (g) Left lobe of stage 27 chick lung, hybridized *in situ* with a probe to detect *cek3* transcripts. *cek3* mRNA was detected in the epithelium. (h) Detection of *cek3* mRNA in the epithelium of the right lobe of stage 32 chick lung. (i) Posthybridization cryosection (50 μm) of a lung hybridized with an antisense *relA* probe. Shown is a cross section through the trachea of a stage 28 lung, confirming that *relA* expression is mesenchymal. (j) Posthybridization cryosection of stage 30 lung hybridized with an antisense *cek3* probe, which hybridized specifically to the developing epithelium. Scale bars: (a) 40 μm, (b) 50 μm, (c) 70 μm, (d, e) 20 μm, (f) 70 μm, (g) 20 μm, (h) 30 μm, (i) 15 μm, and (j) 15 μm.

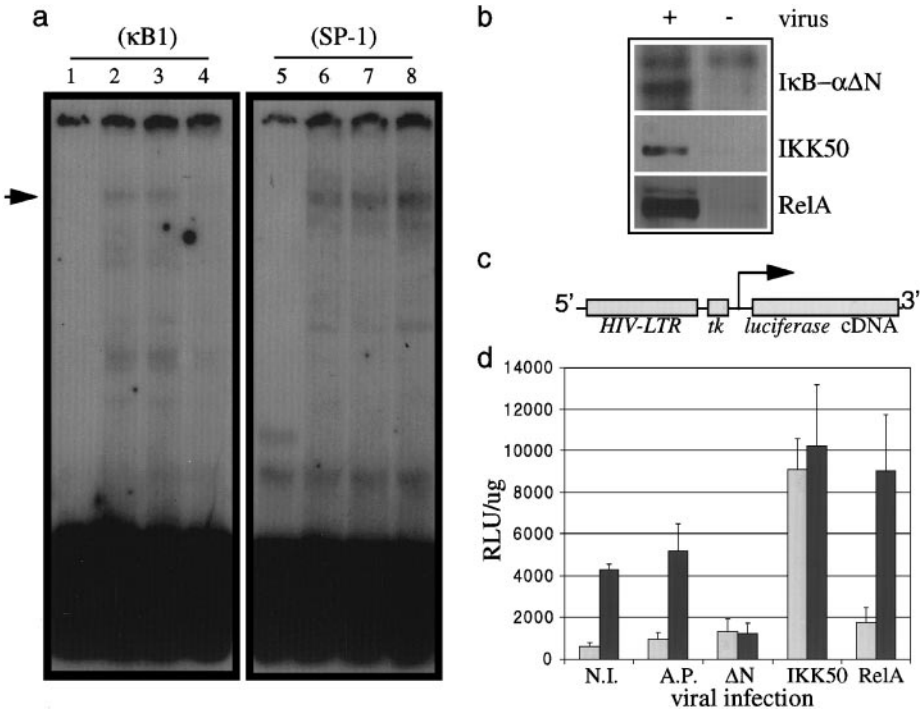

FIG. 2. DNA binding activity of NF-κB is present in nuclear extracts of embryonic chick lungs and can be altered *in vitro* by mutant isoforms of IκB-α and IKK-2. (a) Nuclear extracts harvested from embryonic chick lungs at stage 28 (lanes 2, 5) and stage 32 (lanes 3, 4, 7, 8) produce a mobility shift in a labeled oligonucleotide encompassing the NF-κB binding sites of the HIV-LTR enhancer (κB1; lanes 1–4) or canonical SP-1 binding sites (lanes 5–8). The stage 32 chick lungs were divided into proximal (lanes 3, 7) and distal (lanes 4, 8) elements prior to the isolation of nuclear extracts. Lanes 1 and 4 contain no nuclear extracts. Arrow indicates the NF-κB:DNA complex. (b) Western analysis of whole-cell extracts from chick embryonic fibroblasts that are uninfected (−) or infected (+) with RCAS-αΔN (top), RCAS-IKK50 (middle), and RCAS-RelA (bottom). Membranes were incubated with antibodies directed against mouse IκB-α (top), FLAG epitope (middle), or human p65/RelA (bottom). (c) Schematic structural diagram of the NF-κB-responsive reporter plasmid composed of the NF-κB-dependent segment of the HIV-LTR enhancer, the *tk* promoter, and the *Photinus* luciferase cDNA. (d) Uninfected (N.I.) and infected chick embryonic fibroblasts were transfected with the reporter plasmid. Cells were treated with (dark bars) or without (light bars) 2 ng/ml recombinant human tumor necrosis factor-α, a known stimulator of NF-κB activity. Results are reported as relative light units per μg of total cellular protein. All values represent the average of three independent experiments, and all experiments were performed in triplicate. Error bars represent the standard deviations. Student's *t* test shows the following statistical significance values compared to cells infected with RCAS-alkaline phosphatase: $P < 0.01$ for IκB-αΔN, $P < 0.03$ for IKK50. A.P., infection with RCAS-alkaline phosphatase. ΔN, infection with RCAS-αΔN. IKK50, infection with RCAS-IKK50. RelA, infection with RCAS-RelA.

retrovirus, RCAS-BP(A). RCAS-αΔN, RCAS-IKK50, RCAS-RelA, and RCAS-alkaline phosphatase were produced in chick embryonic fibroblasts. Western analysis was performed on extracts from infected cells at 18 h postinfection to confirm viral infection and production of the proteins of interest (Fig. 2b). An antibody against the C-terminal domain of mouse IκB-α recognized two protein species in cells infected with RCAS-αΔN, one approximately at 40 kDa, the other at approximately 37 kDa. Because IκB-αΔN lacks 40 residues of the wild-type IκB-α protein (Bushdid *et al.*, 1998), it is expected that the upper band represents endogenous IκB-α, while the lower band represents IκB-αΔN. A mouse monoclonal anti-FLAG antibody specifically recognized a single 87-kDa protein species in cells infected with RCAS-IKK50, while not recognizing any proteins from uninfected cells. This is the expected size of the FLAG-tagged IKK50. Cells infected with RCAS-RelA produced a protein species recognized by an antibody against human p65/RelA. The size of this species was about 65 kDa, the expected size of wild-type RelA, and was consistent with the presence of a faint band of the identical size from uninfected cells. These results demonstrate that the viruses used are infectious and are expressing the proteins of interest.

To test the ability of these various proteins to modulate NF-κB activity, CEFs were infected with RCAS-αΔN, RCAS-IKK50, RCAS-RelA, or RCAS-alkaline phosphatase. Infected and uninfected cells were transfected with an NF-κB reporter plasmid composed of the NF-κB-responsive HIV-LTR enhancer, the *tk* promoter, and the *Photinus*

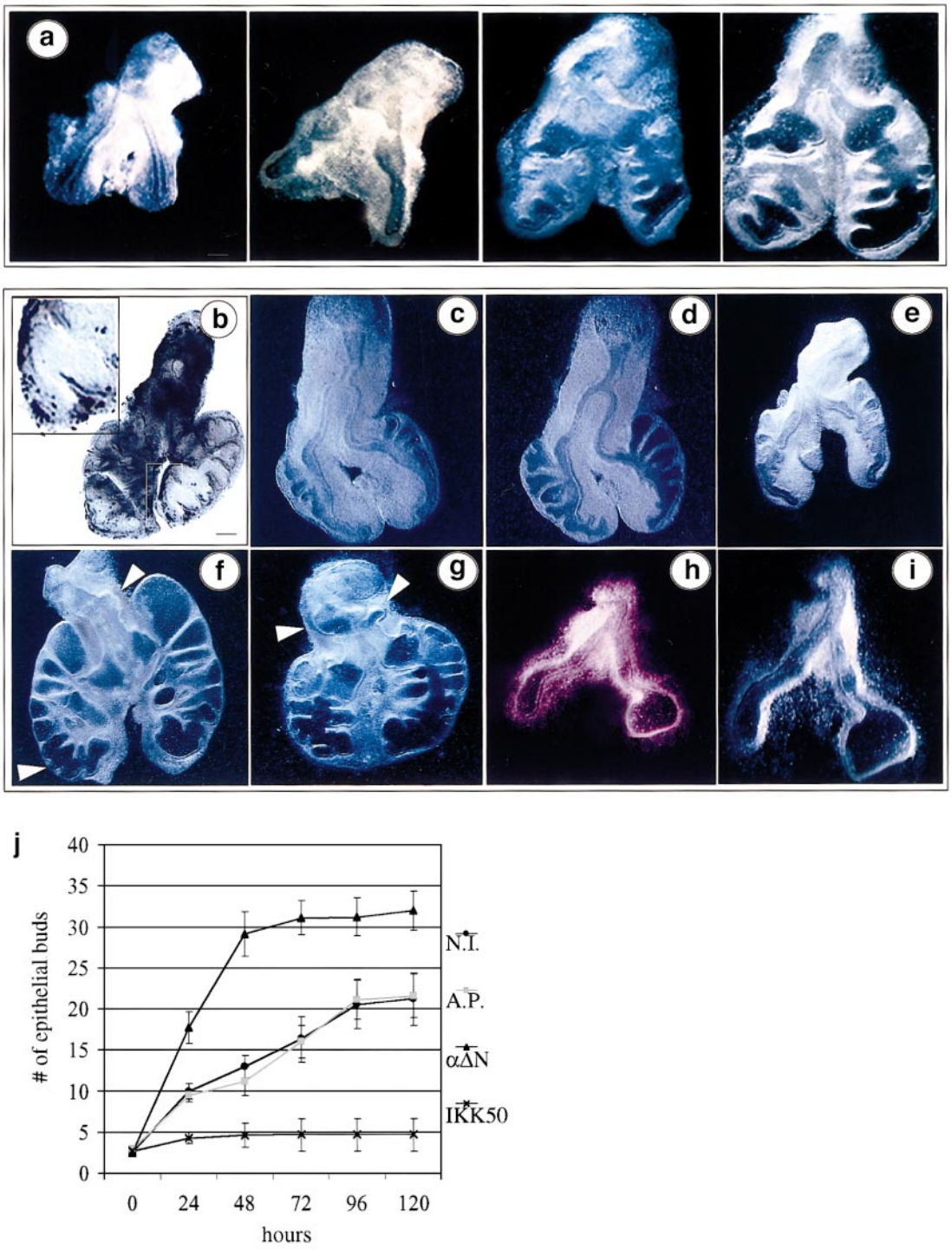

FIG. 3. Inhibition of mesenchymal NF-κB activity increases lung branching, while activation of mesenchymal NF-κB inhibits lung branching. (a–d) Whole organ culture of a single embryonic lung isolated from stage 27 chick embryo and cultured for 72 h. Photographs were taken at 0, 24, 48, or 72 h. Lung cultures infected with RCAS-alkaline phosphatase (b, c, d), RCAS-αΔN (e, f, g), or RCAS-IKK50 (h, i). (b) Viral alkaline phosphatase expression (dark areas) within the lung mesenchyme at 72 h after infection. (c) Lung culture infected with RCAS-alkaline phosphatase after 48 h in culture. (d) The same lung culture shown in (c) after 72 h in culture. (e) Lung cultures infected with RCAS-αΔN after 24 h in culture. (f) The same lung culture shown in (e), after 72 h of culture. Arrows indicate increased distal epithelial bud formation and *de novo* epithelial bud formation in the proximal portions of the lung culture. (g) Another example of lung culture

luciferase cDNA (Fig. 2c). The HIV-LTR enhancer has been shown to be responsive to a wide array of NF-κB complexes (Kretzschmar *et al.*, 1992; Doerre *et al.*, 1993). Therefore, this reporter allows changes in NF-κB activity to be measured as a function of relative light units (RLUs). Infection of cells with RCAS-alkaline phosphatase did not significantly alter the basal level of NF-κB activity nor the induction of NF-κB by TNF-α (Fig. 2d). Although RCAS-αΔN did not alter basal levels of NF-κB activity, the induction of NF-κB was significantly inhibited in these cells. The dominant positive IKK50 increased basal NF-κB activity nearly 20-fold, such that further stimulation was not achieved by the addition of TNF-α. Wild-type RelA increased basal NF-κB activity nearly 2-fold compared to cells infected with RCAS-alkaline phosphatase. TNF-α produced a 4-fold increase in NF-κB activity in RCAS-RelA-infected cells, with 2.25 times more RLUs produced than in control cells treated with TNF-α. Overexpression of RelA would be predicted to increase reporter activity to a lesser extent than IKK50, as RelA activity can be regulated by endogenous IκBs. These results suggest that NF-κB activity can be inhibited by viral expression of IκB-αΔN, and hyperactivated by viral expression of IKK50, while being modestly activated by viral overexpression of RelA.

Whole embryonic chick lungs were cultured over a period of 5 days to reproduce a characteristic pattern of epithelial branching (Fig. 3a). Abrogation of mesenchymal NF-κB activity in these lung cultures was used to examine the potential consequences upon epithelial branching in the embryonic chick lung. Activity of NF-κB was specifically inhibited in the mesenchyme of whole lungs cultured *in vitro*, by infection of lung mesenchyme with RCAS-αΔN. Similar viral delivery of RCAS-alkaline phosphatase was used to control for the effects of viral infection on lung branching and to confirm the expression of virally encoded products. Histochemical staining for alkaline phosphatase activity confirmed the expression of viral gene products in the lung cultures, which appeared to be restricted mainly to the mesenchyme of the infected lung cultures (Fig. 3b). Infection of the mesenchyme with RCAS-alkaline phosphatase did not affect the pattern or level of epithelial branching compared to uninfected lungs after 24 or 72 h in culture (Figs. 3c and 3d). Infection of the lung mesenchyme with RCAS-αΔN increased the amount of epithelial branching, which was evident as early as 24 h after the cultures were established (Fig. 3e). It is intriguing to note that with the interruption of mesenchymal NF-κB activity, there appeared to be novel bud formation in the normally nonbudding epithelial structures of the lung after 72 h in culture, such as in the trachea and the primary bronchioles (Figs. 3f and 3g, arrows). Uninfected lung cultures, or those infected with RCAS-alkaline phosphatase, did not form any proximal epithelial buds. Examination of the total number of lung buds demonstrated that expression of IκB-αΔN in the lung mesenchyme resulted in a significant increase in the number of epithelial buds that were present after 5 days in culture compared to lungs expressing alkaline phosphatase (Fig. 3j).

In order to determine the consequences of an increase in mesenchymal NF-κB activity, NF-κB was constitutively activated in the mesenchyme of whole lung cultures by infection with RCAS-IKK50. After 4 days in culture, mesenchymal expression of IKK50 caused a severe reduction in the budding of the epithelium, compared to lungs expressing alkaline phosphatase (Figs. 3h and 3i). Although the inhibition of epithelial budding by expression of IKK-50 in the mesenchyme was observed as early as 24 h, it was maintained over a period of 5 days, thus it does not appear to represent a delay in the formation of epithelial buds. The number of epithelial buds was quantitated daily for 5 days. Lungs infected with RCAS-αΔN consistently developed a greater number of epithelial buds, while those infected with RCAS-IKK50 formed fewer lung buds than control lungs (Fig. 3j; $n = 10$ for each viral infection). These results suggest that increased NF-κB activity in the mesenchyme of the developing chick lung may ultimately repress bud formation in the adjacent epithelium.

The effect of mesenchymal NF-κB activity on cellular proliferation in the developing lung was determined. Whole lung cultures in which mesenchyme was infected with RCAS-αΔN or RCAS-IKK50 were examined for genomic incorporation of the nucleotide analog BrdU. BrdU incorporation was detected in uninfected lung cultures (Fig. 4a). A similar pattern was observed in lung cultures infected with RCAS-alkaline phosphatase, in which BrdU incorporation is observed in the epithelium and in the adjacent mesenchymal condensations (Fig. 4b, arrow). Incorporation of BrdU in lung cultures expressing IκB-αΔN was evident in the region of the epithelium that appeared to be in the early stages of bud formation (Figs. 4c and 4d), similar to the pattern of BrdU incorporation that was observed in lungs infected with RCAS-alkaline phosphatase. However, the

infected with RCAS-αΔN, after 72 h of culture. Arrows indicate *de novo* epithelial bud formation in the proximal areas of the lung. (h) Lung cultures infected with RCAS-IKK50 after 24 h and (i) 96 h of culture. (j) Quantification of epithelial bud formation in uninfected cultured lungs or cultured lungs infected with RCAS-alkaline phosphatase ($n = 10$), RCAS-αΔN ($n = 10$, $P < 0.005$), or RCAS-IKK50 ($n = 10$, $P < 0.005$). The number of epithelial buds was counted upon isolation ($t = 0$), then counted again at 24-h intervals for a total of 72 h. The final budding index was calculated as [(final number of buds) − (initial number of buds)]. Each value presented represents the mean of 10 individually infected and cultured lungs, while error bars indicated the standard deviation. Scale bar, 50 μm for all images except inset, in which scale bar represents 20μm. N.I., not infected. A.P., infection with RCAS-alkaline phosphatase. αΔN, infection with RCAS-αΔN. IKK50, infection with RCAS-IKK50.

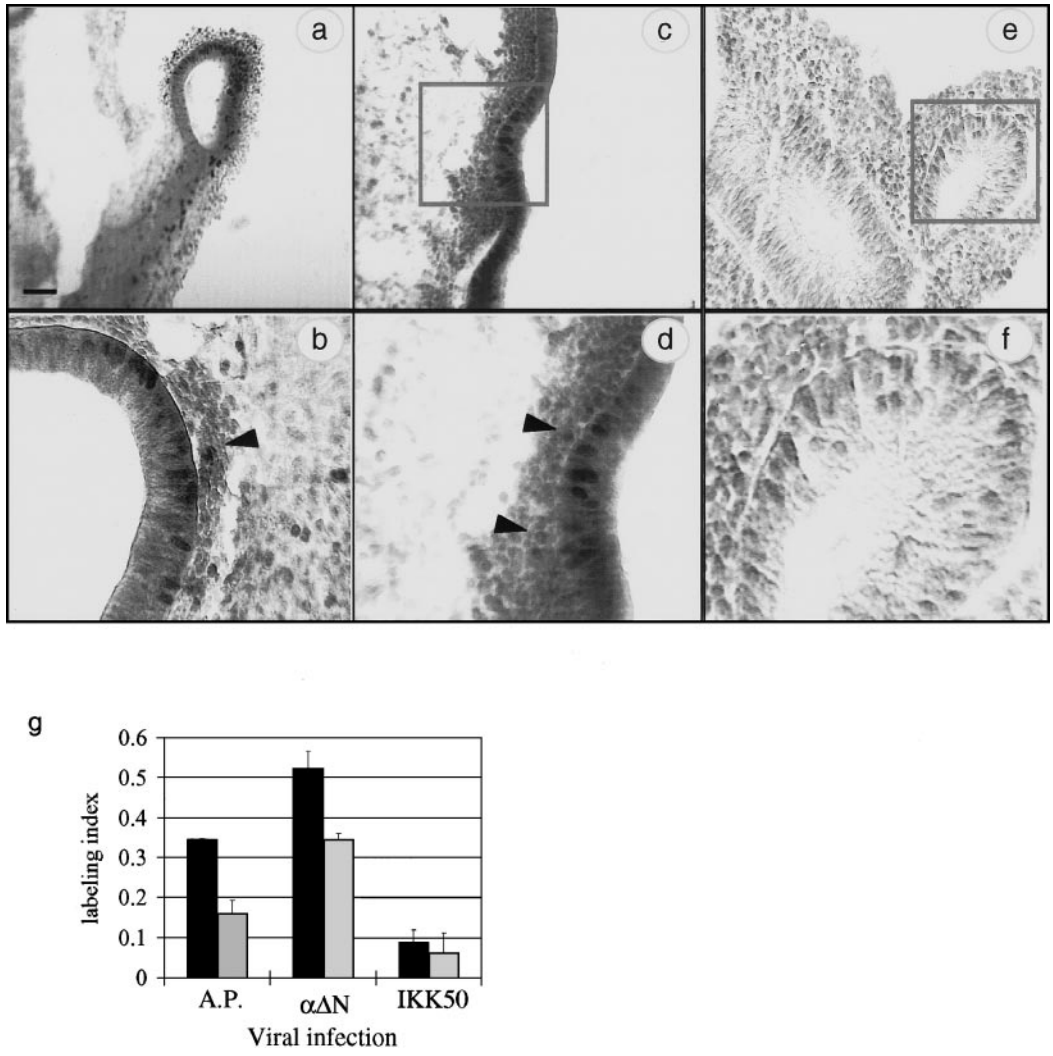

FIG. 4. NF-κB inhibits proliferation of epithelial and mesenchymal embryonic lung cells in organ culture. Uninfected lung cultures (a) or lung cultures infected with RCAS-alkaline phosphatase (b), RCAS-αΔN (c, d), or RCAS-IKK50 (e, f) were cultured for 48 h. BrdU was added to the culture media for the final 6 h of culture. BrdU incorporation was detected by whole-mount immunohistochemistry of fixed tissue. Shown are representative 7-μm cryosections; scale bar represents 30 μm (a, c, e) and 10 μm (b, d, f). Boxed areas indicate areas of higher magnification in the adjacent panel. Arrows indicate examples of proliferative cells within the mesenchymal condensation. (g) Quantification of BrdU incorporation in lungs infected with RCAS-alkaline phosphatase, RCAS-αΔN ($P < 0.03$), or RCAS-IKK50 ($P < 0.01$). The number of epithelial cells (dark bars) or mesenchymal cells (light bars) in a single 40× field that were labeled with BrdU were counted and compared to the total number of cells in that field. The labeling index was calculated as follows: [(number of labeled cells)/(total number of cells)]. Each value represents the average of 10 fields from each of three individually infected and cultured lungs, while error bars indicate standard deviations. A.P., infection with RCAS-alkaline phosphatase. αΔN, infection with RCAS-αΔN. IKK50, infection with RCAS-IKK50.

proportion of BrdU-positive staining cells per total number of epithelial cells was higher in lungs expressing IκB-αΔN than in lungs expressing alkaline phosphatase. Upon histological examination, the mesenchymal condensations in direct apposition to the proliferating epithelium were more pronounced in the lung cultures infected with RCAS-αΔN, and these mesenchymal condensations contained more BrdU-labeled cells in the RCAS-αΔN-infected lungs compared to control lungs. In contrast, RCAS-IKK50 sharply inhibited BrdU incorporation into the epithelium and mesenchyme (Figs. 4e and 4f). Furthermore, there was a marked absence of mesenchymal condensations adjacent to the epithelial layer of cells in lungs infected with RCAS-IKK50. These results suggest that activation of NF-κB in the lung mesenchyme may function to inhibit the mesenchymal condensation required prior to the formation of an epithe-

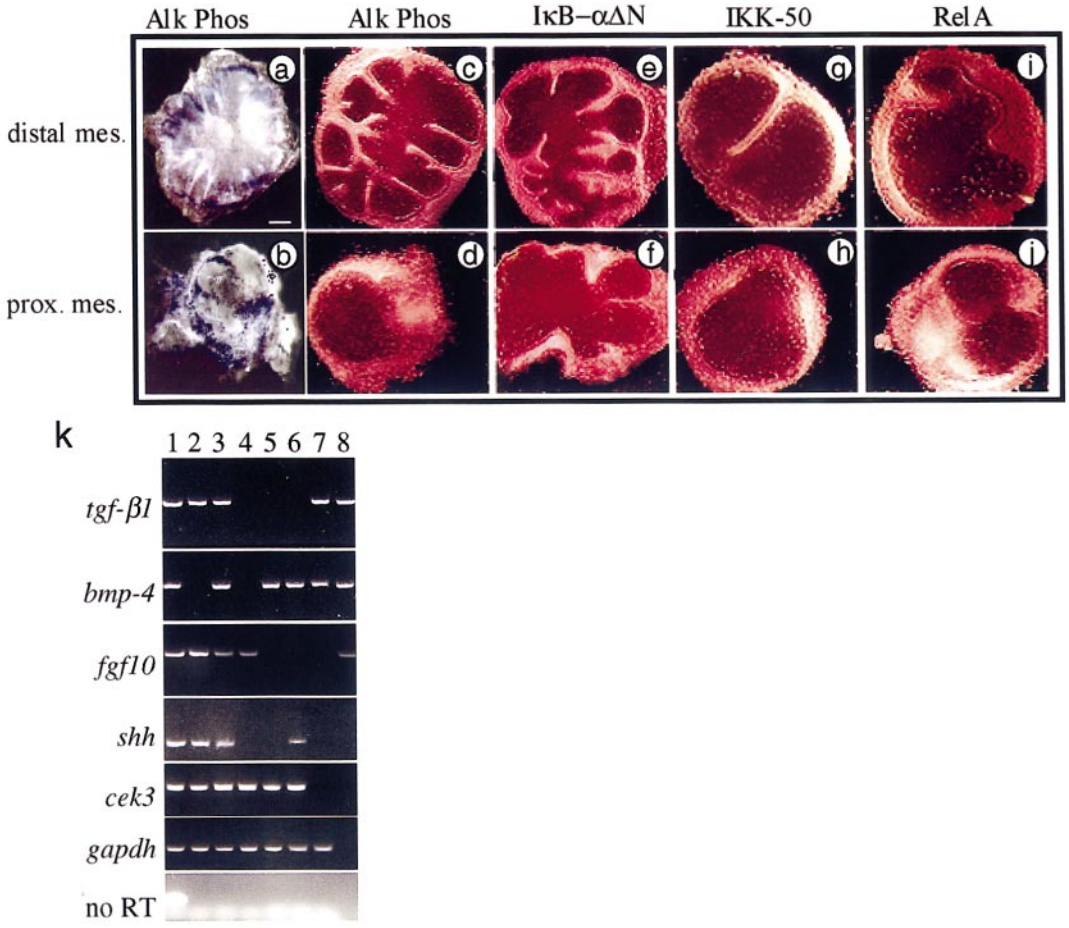

FIG. 5. Distal mesenchyme (a, c, e, g, i) or proximal mesenchyme (b, d, f, h, j) was isolated from stage 27 chick embryos, infected with RCAS-alkaline phosphatase (a–d), RCAS-αΔN (e, f), RCAS-IKK50 (g, h), or RCAS-RelA (i, j), and then recombined with distal epithelium from stage 27 chick embryos. Recombinations were cultured on filters for a total of 5 days and then photographed. Histochemical staining for alkaline phosphatase activity was performed on recombinants in which the distal or proximal mesenchyme was infected with RCAS-alkaline phosphatase (a, b). Scale bar, 25 μm. Alk Phos, mesenchyme infected with RCAS-alkaline phosphatase. IκB-αΔN, mesenchyme infected with RCAS-αΔN. IKK-50, mesenchyme infected with RCAS-IKK50. RelA, mesenchyme infected with RCAS-RelA. (k) RT-PCR analysis of mRNA isolated from freshly harvested stage 25 (lane 1) or stage 30 (lane 2) embryonic chick lungs or from stage 27 lungs that have been cultured for 24 (lane 3) or 72 (lane 4) h. Stage 27 lungs were separated into proximal epithelium (lane 5), distal epithelium (lane 6), proximal mesenchyme (lane 7), and distal mesenchyme (lane 8) and used for RT-PCR analysis. PCR was performed using primer pairs that would specifically amplify cDNA sequences corresponding to *tgf-b1, bmp-4, fgf10, shh, fgfr2,* and *gapdh.* Reverse transcription was left out of the reaction (bottom, no RT) for lanes 2–8, while lane 1 shows amplification of GAPDH as a positive control.

lial bud, while decreasing cellular proliferation in the lung epithelium and mesenchyme.

In an attempt to understand the precise role of NF-κB in the proximal versus the distal embryonic lung mesenchyme, reciprocal recombination experiments were performed, in which mesenchyme was isolated from the proximal or the distal portion of stage 28 embryonic chick lungs and cultured with distal epithelium (Fig. 5). In these experiments, mesenchyme was infected with the RCAS viruses prior to recombination with the epithelium. In recombinants infected with RCAS-alkaline phosphatase, histochemical staining for alkaline phosphatase activity demonstrated that infection was limited to the mesenchyme and that expression of viral products occurred similarly in proximal and distal mesenchyme (Figs. 5a and 5b). Consistent with classical recombination experiments, the distal mesenchyme infected with RCAS-alkaline phosphatase induced budding when recombined with the epithelium (Fig. 5c); proximal mesenchyme infected with RCAS-alkaline phosphatase inhibited budding of the epithelium (Fig. 5d). These results confirm that viral infection and expression of a control gene product in the mesenchyme have no effect on

epithelial budding. NF-κB activity was selectively inhibited in the proximal or distal mesenchyme by infection with RCAS-αΔN. Inhibition of NF-κB activity in the proximal mesenchyme by expression of IκB-αΔN blocked the repressive effects of the proximal mesenchyme on budding of the epithelium (Figs. 5e and 5f). Expression of IκB-αΔN in the distal mesenchyme did not alter the high amount of epithelial budding produced by the distal mesenchyme.

The consequence of increased NF-κB activity in the distal or proximal mesenchyme was investigated using this same recombination system. RCAS-IKK50 in the distal mesenchyme strongly inhibited the induction of epithelial buds by the distal mesenchyme, while expression of IKK50 in proximal mesenchyme did not alter the inhibition of epithelial budding that is normally observed with proximal mesenchyme (Figs. 5g and 5h). In order to determine specifically whether RelA is involved in the repression of epithelial branching by the proximal mesenchyme, distal or proximal mesenchyme was infected with RCAS-RelA. The expression of RelA in the distal mesenchyme was sufficient to inhibit epithelial bud formation by distal mesenchyme after 72 h in culture (Fig. 5i). Overexpression of RelA in the proximal mesenchyme had no effect on the embryonic chick lung epithelium beyond the inhibition of epithelial budding by proximal mesenchyme (Fig. 5j). These results suggest that NF-κB activity in the proximal mesenchyme may be critical for limiting epithelial budding in the developing trachea.

To confirm the separation of the epithelium from the mesenchyme, the expression of epithelial and mesenchymal markers was examined using reverse transcription-polymerase chain reaction analysis of total cellular RNA isolated from the proximal or distal epithelium or mesenchyme. *cek3/fgfr2* was expressed at stage 25 and stage 30 in intact lungs and also in intact cultured lungs (Fig. 5k). Upon separation of the epithelium from the mesenchyme, *cek3* was found in the proximal and distal epithelium, but not in mesenchyme. *Tgf-β1* mRNA was detected in both proximal and distal mesenchyme, but not in the epithelium, confirming the complete separation of epithelium from mesenchyme. These results also suggest that separation of epithelium from mesenchyme did not alter the expression profile of *tgf-β1, fgf10, bmp-4,* and *shh*. Furthermore, lungs cultured under the conditions described herein expressed *fgf10, bmp-4, tgf-β1, shh,* and *cek3/fgfr2* in a manner that was consistent with freshly harvested, intact lungs at approximately equivalent stages of development.

The expression of several potential lung morphogens was examined by whole-mount *in situ* hybridization in epithelial–mesenchymal recombinations in which the mesenchyme was uninfected or infected with RCAS-αΔN or RCAS-IKK50. Uninfected distal mesenchyme did not express detectable levels of *tgf-β1* mRNA (Fig. 6a). Distal mesenchyme infected with RCAS-αΔN also did not express *tgf-β1* mRNA (data not shown). However, when distal mesenchyme was infected with RCAS-IKK50, intense *tgf-β1* expression was induced in the mesenchyme (Fig. 6a).Uninfected distal mesenchyme recombined with distal epithelium expressed significant levels of *fgf10* mRNA (Fig. 6b). This expression appeared in discrete locations in the mesenchyme that was adjacent to branching epithelium, consistent with previous descriptions of murine *fgf10* expression (Bellusci *et al.*, 1998). Distal mesenchyme that was infected with RCAS-IKK50 did not express *fgf10* mRNA in the mesenchyme (Fig. 6b). Expression of the genes encoding *shh, fgf7, wnt7a,* or *hoxB3* were not appreciably altered in lungs infected with RCAS-IKK50 compared to uninfected lung cultures (Fig. 6c and data not shown).

Interestingly, there was little *fgf10* mRNA expression in distal lung mesenchyme that had been infected with RCAS-αΔN, despite the high level of epithelial budding that was observed (Fig. 6f). Of significant interest, expression of *bmp-4* was dramatically induced in distal mesenchyme infected with RCAS-αΔN, but not in uninfected distal mesenchyme (Fig. 6d). Expression of *bmp-4* in the distal epithelium occurred regardless of whether the epithelium was recombined with uninfected distal mesenchyme or with distal mesenchyme infected with RCAS-αΔN. Expression of IKK50 in the distal mesenchyme did not affect the epithelial expression of *bmp-4* as determined by whole-mount *in situ* hybridization (data not shown). Mesenchymal expression of the gene encoding the chick homologue of *twist* (*twi*) was completely inhibited in lung cultures that were infected with RCAS-αΔN (Fig. 6e). Expression of the genes encoding *shh, fgf7, hoxb3,* or *wnt7a* was not altered by expression of IκB-αΔN in the distal mesenchyme.

DISCUSSION

The role of the embryonic lung mesenchyme in directing the branching morphogenesis of the lung epithelium has been well established (Rudnick, 1933; Alescio and Cassini, 1962; Wessells, 1970; Shannon *et al.*, 1994). Here we provide evidence that the transcription factor NF-κB is a critical component of the process by which the mesenchyme can actively limit the growth and branching of the adjacent epithelium. The importance of transcription factors in branching lung morphogenesis has long been recognized, particularly in regard to the positive induction of growth and branching. For example, *n-myc*, a transcription factor of the basic leucine zipper family, is expressed exclusively in the lung epithelium, and targeted disruption of the *n-myc* gene in mice interrupts lung development past the initial bifurcation of the lung epithelium (Sawai *et al.*, 1993; Charron *et al.*, 1992; Stanton *et al.*, 1992). Significantly less is known regarding the transcription factors that drive the expression of developmentally significant lung morphogens in the embryonic lung mesenchyme, particularly in the proximal lung mesenchyme. Expression of several members of the homeobox family of transcription factors has been described, such as *hoxb3,* which is expressed throughout the entire embryonic murine lung mesenchyme beginning at E10.5 (Rex *et al.*, 1994; Walters *et al.*,

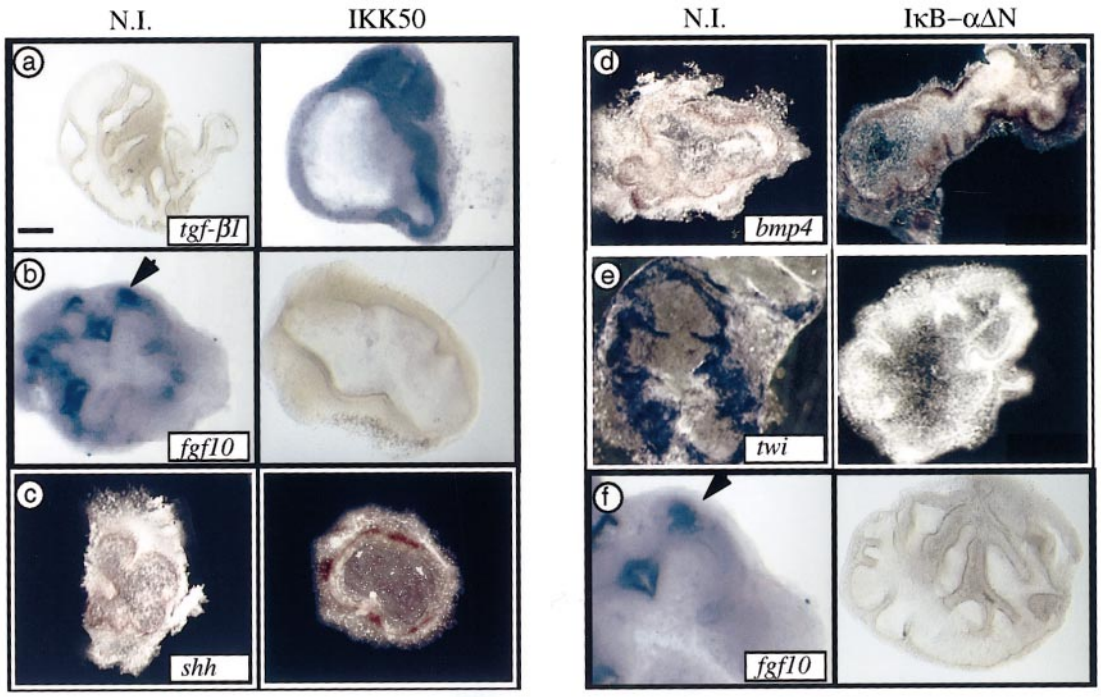

FIG. 6. NF-κB represses the expression of avian *bmp-4* and *fgf10*, but activates expression of *tgf-β1* and *twist*. Distal mesenchyme from stage 27 chick embryos was infected with RCAS-IKK50 (right sides of a, b, c) or RCAS-αΔN (right sides of d, e, f) and then recombined with distal epithelium for 3 days of culture. Recombinants were subjected to whole-mount *in situ* hybridization to determine the relative transcript levels of *tgf-b1* (a), *fgf10* (b, f), *shh* (c), *bmp-4* (d), *twist* (e), *wnt7a, fgf7,* and *hoxb3* (not shown). Arrows indicate discrete domains of *fgf10* mRNA expression. Scale bar, 40 μm. N.I., not infected. IKK50, mesenchyme infected with RCAS-IKK50. IκB-αΔN, mesenchyme infected with RCAS-αΔN.

1997). Although the expression pattern is known, the functional significance of this expression has yet to be determined. NF-κB represents the first transcription factor to be identified within the proximal lung mesenchyme that controls transcriptional events that negatively regulate growth and branching of the adjacent epithelium.

This hypothesis is supported by results presented herein showing that increased mesenchymal NF-κB activity results in inhibition of epithelial branching, shown using two different methods. First, infection of the mesenchyme of whole lung cultures was achieved by bathing the lungs in concentrated viral stock of RCAS-IKK50. It is inferred that expression of IKK50 was localized to the mesenchyme of the cultured lungs, based on this mode of infection. The examination of alkaline phosphatase expression in whole-lung cultures infected with RCAS-alkaline phosphatase confirmed that expression of viral gene products was restricted to the mesenchyme of the infected whole-lung cultures. Furthermore, when the mesenchyme was separated from the epithelium, then was infected with RCAS-IKK50, and later was recombined with the epithelium, similar results that demonstrated a loss of epithelial budding due to increased NF-κB activity in the mesenchyme were obtained.

Mesenchymal NF-κB also appears to regulate proliferation in the embryonic chick lung. Elevated NF-κB activity caused cellular proliferation to dramatically decrease, in both the epithelium and the mesenchyme. Histologic characteristics of the embryonic lung mesenchyme, such as cellular proliferation and condensation at the distal points of epithelial buds, were disrupted by increased NF-κB activity. The elevated NF-κB activity may interfere with the formation of the condensations of the distal mesenchyme, which impairs the ability of the distal mesenchyme to induce proliferation of the underlying epithelium. This hypothesis is supported by the reciprocal recombination experiments, in which inhibition of NF-κB activity by IκB-αΔN abrogated the repressive effects of the proximal mesenchyme on the budding of the distal epithelium. Furthermore, decreased NF-κB activity in the lung mesenchyme resulted in increased proliferation of the mesenchyme and the formation of mesenchymal condensations adjacent to the lung epithelium. This may explain why epithelial proliferation was increased when NF-κB activity was inhibited in the lung mesenchyme.

As NF-κB is a transcription factor, it is likely that NF-κB influences the expression of genes that are synthesized in the mesenchyme, which then exert their influence on the adjacent epithelium. The pattern of *relA* expression is reminiscent of the expression pattern of TGF-β1, a secreted signaling

molecule that negatively regulates epithelial branching in the murine lung. This led to the hypothesis that *relA* may be involved in establishing or maintaining nonbranched epithelial structures in the developing lung by negatively regulating epithelial branching, perhaps through regulation of *tgf-β1* expression. TGF-β1 is normally expressed in the proximal mesenchyme but in the distal mesenchyme, TGF-β1 is expressed only in the mesenchyme adjacent to cleft points in the branching epithelium (Heine *et al.*, 1990; Pelton *et al.*, 1991). Ectopic expression of TGF-β1 in the distal epithelium via the *SP-C* promoter/enhancer results in neonatal lethality associated with decreased saccule formation, hypoplasticity, and a block in the differentiation of distal cell types (Zhao *et al.*, 1996). The TGF-β1 receptors are expressed on the embryonic lung epithelium, and when activated by TGF-β1 at the epithelial cell surface, transduce their signal to Smad proteins, intracellular signaling molecules that function in the TGF-β/BMP pathway (Derynck and Zhang, 1996). Abrogation of Smad2 and Smad3 together, or abrogation of Smad4, was shown to increase epithelial branch formation in organ culture, further evidence that TGF-β1 signaling inhibits epithelial branch formation (Zhao *et al.*, 1998). Experiments herein demonstrate that *tgf-β1* expression can be induced in the distal mesenchyme by elevation of mesenchymal NF-κB activity. This induction of *tgf-β1* expression by hyperactivation of NF-κB activity in organ cultures correlates with an inhibition of epithelial proliferation and inhibition of epithelial budding. These epithelial phenomena are also observed when murine lungs are cultured in the presence of exogenous TGF-β1 (Serra *et al.*, 1994). The induction of mesenchymal *tgf-β1* expression by increased NF-κB activity may be one mechanism by which mesenchymal NF-κB influences the adjacent epithelium. Although NF-κB is known to modulate the expression of target genes, the data presented herein do not determine whether the effect of NF-κB on *tgf-β1* expression is direct or indirect.

Other molecules that are known to regulate branching morphogenesis in the mouse, rat, or chick lung include FGFs and BMPs. For example, FGF-10 greatly enhances epithelial bud formation in lung cultures, acting as a chemotactic factor for lung epithelial cells (Bellusci *et al.*, 1997; Park *et al.*, 1998; Hogan, 1999). Gene targeting experiments that generated *fgf10*-deficient mice showed that induction of the lung bud from the embryonic foregut occurs even in the absence of *fgf10*, but no subsequent epithelial branching takes place. Because of this alteration in the genetic specification of lung morphogenesis, the *fgf10*-deficient mice have a normally developed trachea, but lack features distal to the trachea, including lobes and bronchi (Park *et al.*, 1998; Min *et al.*, 1998; Sekine *et al.*, 1999). Interestingly, results presented in this study show that hyperactivation of mesenchymal NF-κB activity in organ culture causes a striking reciprocal inhibition of *fgf10* expression in the embryonic lung mesenchyme. The decrease in *fgf10* expression correlates with a block in epithelial budding due to increased NF-κB activity. This suggests that one mechanism by which mesenchymal NF-κB activity influences epithelial branching is the transcriptional regulation of factors that are synthesized in the mesenchyme, which then act upon the epithelium. NF-κB-mediated inhibition of the factors that induce branching in the lung, such as *fgf10*, may prevent precocious branching and differentiation of the lung epithelium, while maintaining the outgrowth of the trachea and bronchi.

It is intriguing that *fgf10* is also required for limb development in mice, as are *fgf2, fgf4,* and *fgf8* (Fallon *et al.*, 1994; Niswander *et al.*, 1993; Vogel *et al.*, 1996; Crossley *et al.*, 1996). FGF-2, -4, and -8 are produced in the AER of the developing limb bud and are thought to act upon the embryonic limb mesenchyme, directing proximal to distal outgrowth of the developing limb. FGF-10, however, is produced in the lateral plate mesoderm within the prelimb field immediately prior to limb bud initiation (Ohuchi *et al.*, 1997). These observations are consistent with a role for *fgf10* in the induction of limb development. Indeed, mice that are *fgf10*-deficient do not form any type of limb structure (Min *et al.*, 1998; Sekine *et al.*, 1999). Previous studies in chick and mouse models have shown that NF-κB may also be important for limb development, and limb development is impaired in mice that carry a targeted deletion of the *IKK1* gene (Hu *et al.*, 1999; Takeda *et al.*, 1999). Because loss of *IKK1* would greatly decrease the activity of a broad range of NF-κB complexes in several tissues, this suggests that NF-κB, like FGF-10, is important for limb morphogenesis. There are many parallels in the genetic pathways that regulate limb and lung development, particularly in regard to genes involved in epithelial–mesenchymal interactions. It may not be surprising, therefore, that these studies suggest a correlation between the roles of NF-κB and *fgf10* in the development of both the limb and the lung.

In our studies, mesenchymal expression of *fgf10* was not greatly altered by the inhibition of NF-κB activity. It might have been expected that the increase in branching that is observed with the inhibition of NF-κB activity would correlate with an increase in *fgf10* mRNA expression. One possible explanation for this observation is based on the fact that *fgf10* expression in the developing murine lung is very transient, appearing initially in a region of mesenchyme that is at a novel branch point of the distal epithelium. Epithelial cells form a bud as they migrate toward the source of FGF-10. As the epithelial cells approach *fgf10*, the expression of *fgf10* is turned off, perhaps in response to signals received from the approaching epithelium. This causes *fgf10* expression to appear in two divergent, smaller domains of mesenchyme, inducing a bifurcated migration of the epithelium (Bellusci *et al.*, 1997; Hogan, 1999). Because *fgf10* expression is very dynamic and transient, inhibition of NF-κB in the lung mesenchyme may increase the rate at which *fgf10* expression turns on and off. This hypothesis is supported by the observation that budding of the embryonic lung epithelium occurs more quickly when mesenchymal NF-κB activity is inhibited with IκB-αΔN. In whole-lung cultures, quantification of lung buds at 24-h

intervals showed that control lungs increased the number of epithelial buds for 72 h, while lungs infected with RCAS-αΔN increased the numbers of lung buds within the first 48 h of culture. In the studies presented here, expression of *fgf10* mRNA was examined only after 72 h in culture, and it is therefore possible that any transient increase in *fgf10* expression may have turned off prior to analysis of the tissue.

The mesenchymal expression of *bmp-4* was greatly increased by the inhibition of NF-κB activity in our *ex vivo* lung cultures, which suggests that NF-κB actively limits the expression of *bmp-4*. This is intriguing in light of the fact that the *Drosophila* homologue of NF-κB, *dorsal,* is known to regulate the *Drosophila bmp* homologue, *decapentaplegic* (*dpp*), early in embryogenesis (Huang *et al.,* 1993; Schwyter *et al.,* 1995). The inverse relationship between NF-κB activity and *bmp-4* expression was previously observed in the developing chick limb (Bushdid *et al.,* 1998; Kanegae *et al.,* 1998), suggesting that one of the major developmental targets of NF-κB may be the *bmp-4* gene. This may be critical in developing organ systems that rely on precise mesenchymal–epithelial interactions in order to establish appropriate growth patterns, such as the limb and the lung. Inhibition of mesenchymal *bmp-4* gene expression by NF-κB may be one strategy used by the lung to negatively regulate cell growth and branching during lung development. Normally, expression of *bmp-4* mRNA is observed primarily in the distal epithelium of the embryonic lung, with much lower levels of *bmp-4* expression in the distal mesenchyme (Bellusci *et al.,* 1996). Although overexpression of *bmp-4* in the distal epithelium inhibits proliferation of the epithelium, an increase in mesenchymal cell proliferation was observed due to overexpression of *bmp-4* in the epithelium (Bellusci *et al.,* 1996). The function of mesenchymal *bmp-4* is largely unknown, but the striking induction of *bmp-4* expression in lungs infected with IκB-αΔN may account for the increase in mesenchymal cell proliferation and the formation of mesenchymal condensations at epithelial branch points. It is interesting to note that FGF-10 is thought to induce expression of *bmp-4*, at least within the distal epithelial tips (Sekine *et al.,* 1999; Bellusci *et al.,* 1996). Although an increase in *fgf10* expression could account for an increase in *bmp-4* expression, the precise mechanism by which RCAS-αΔN induces and maintains *bmp-4* expression is unknown.

Similar to experiments performed in the chick limb, expression of *twist* was completely inhibited in embryonic lung cultures infected with IκB-αΔN. In early *Drosophila* embryos, *twist* expression is directly regulated by the NF-κB homologue, *dorsal* (Jiang *et al.,* 1991; Pan *et al.,* 1991). The function of *twist* in the developing lung remains largely unknown, as mice that carry targeted deletions in the *twist* gene do not develop beyond embryonic day 9, prior to the onset of lung development (Gitelman *et al.,* 1997). However, expression of murine *twist* has been demonstrated in the mesenchyme of several developing organs that rely on epithelial–mesenchymal interactions, including the lung and the limb, and the human *twist* cDNA was cloned from a lung fibroblast cDNA library, indicating its expression in the human lung mesenchyme. This is consistent with our observation that *twist* is highly expressed in the mesenchyme of the developing chick lung and that this expression can be completely inhibited by eliminating NF-κB activity. Despite the high level of *twist* expression in the mesenchyme of control lung cultures, branching morphogenesis occurs in the IκB-αΔN-mediated absence of *twist* expression. Therefore, the role of *twist* in the mesenchyme of the embryonic lung may not be related to branching morphogenesis, but *twist* might serve other vital roles in the developing lung, an idea that would require further exploration.

The overall pattern of gene expression that is observed upon alterations of NF-κB activity has led to the following model of the proposed role of NF-κB in the developing lung mesenchyme. Mesenchymal expression and activity of NF-κB within the proximal areas of the lung increase expression of *tgf-β1*. TGF-β1 may signal to the epithelium, decreasing proliferation and inhibiting epithelial budding. Proximal activity of NF-κB could also repress expression of *fgf10* within the mesenchyme, an additional means of preventing budding in the proximal epithelium. As FGF-10 acts upon the epithelium to induce budding and to induce expression of *bmp-4*, inhibition of *fgf10* expression by NF-κB may also result in repression of *bmp-4* expression in the adjacent epithelium. Therefore, the role of RelA in the proximal lung mesenchyme may be to modulate transcription within the mesenchyme, the result of which is a decrease in epithelial budding and proliferation. This is not to say that NF-κB is not directly involved in the morphogenesis or differentiation of the embryonic lung epithelium. In fact, preliminary studies suggest that several NF-κB factors may in fact be expressed within the embryonic mouse lung epithelium, although RelA is not among these (unpublished observations, R.S.M.). These observations, and the results presented herein, are evidence that the role of NF-κB in the development of the embryonic lung epithelium begs further investigations. Based on the evolutionary conservation of genetic programs directing lung development from *Drosophila* to mice to humans, it may soon be possible to thoroughly understand the role of NF-κB in the complex genetic pathways of lung development.

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